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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/009,085	07/11/2002	David W. Sammons	6033-001	6300

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EXAMINER	
GABEL, GAILENE	
ART UNIT	**PAPER NUMBER**
1641	

DATE MAILED: 10/14/2005

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	10/009,085	SAMMONS ET AL.	
	Examiner	Art Unit	
	Gailene R. Gabel	1641	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *04 December 2001*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-10* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-10* is/are rejected.

7)☒ Claim(s) *4* is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on *04 December 2001* is/are: a)☐ accepted or b)☒ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

Claims Under Prosecution

1. Claims 1-10 are pending. All claims 1-10 are under examination.

Priority

2. Applicant's claim for the benefit of a prior-filed application under 35 U.S.C. 119(e) or under 35 U.S.C. 120, 121, or 365(c) is acknowledged. The prior-filed application is US Provisional Patent Application Serial No. 60/137,692, which was filed on June 4, 1999 upon which the instant application is a continuation-in-part of.

It is noted that the subject matters of 1) enriching nucleated fetal erythrocytes by charge flow separation to remove a major fraction of platelets and mature erythrocytes without substantial loss of nucleated erythrocytes (recited in claims 2, 3, and 4); and 2) storing digitized images and plurality of coordinates onto a web-based internet server for remote access and manipulation of the stored digitized images (recited in claim 1) are not disclosed and described in ASN 60/137,692 from which the benefit of priority is claimed. As these subject matters are only disclosed in the instant National Stage application, for purposes of priority, claims 1-4 have been granted a priority date of July 11, 2002, which is the "filing fate" at which the last of the 35 USC 371 requirements of this National Application is met by Applicant and received by the Office. As subject matters encompassing histochemical staining, phenotype labeling, karyotype labeling, detection, and imaging are disclosed and described in the provisional application and

recited in claims 5-10 in this National Stage application, claims 5-10 will have the benefit of priority of the provisional application SN 60/137,692 having a filing date of June 4, 1999.

Drawings

3. The drawings are objected to because the legend defining Figure 14 is misspelled, i.e. "Cell Imag". It should be changed to "Cell Image". Corrected drawing sheets in compliance with 37 CFR 1.121(d) are required in reply to the Office action to avoid abandonment of the application. Any amended replacement drawing sheet should include all of the figures appearing on the immediate prior version of the sheet, even if only one figure is being amended. The figure or figure number of an amended drawing should not be labeled as "amended." If a drawing figure is to be canceled, the appropriate figure must be removed from the replacement sheet, and where necessary, the remaining figures must be renumbered and appropriate changes made to the brief description of the several views of the drawings for consistency. Additional replacement sheets may be necessary to show the renumbering of the remaining figures. Each drawing sheet submitted after the filing date of an application must be labeled in the top margin as either "Replacement Sheet" or "New Sheet" pursuant to 37 CFR 1.121(d). If the changes are not accepted by the examiner, the applicant will be notified and informed of any required corrective action in the next Office action. The objection to the drawings will not be held in abeyance.

Claim Rejections - 35 USC § 112

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

4. Claims 1-10 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claim 1 is vague and indefinite because it is unclear as to whether the step of, "obtaining a maternal whole blood sample ..." is intended to be part of the claimed method. As written, it does not appear to have been incorporated as part of the preamble; however, no identifying designation, i.e. step a) or step b), has been assigned thereto. Alternatively, it appears that it should be part of the claimed method as the recitation provides an actual active method step of a procedure.

Claim 1, step c) has improper antecedent basis problem in reciting, "with detectable label". Change to "with the detectable label" for proper antecedent basis.

Claim 1, step d) has improper antecedent basis problem in reciting, "labeled nucleated fetal erythrocytes". Change to "the labeled nucleated fetal erythrocytes" for proper antecedent basis.

Claim 1 is vague and indefinite in reciting, "A prenatal diagnostic method" in the preamble, because it is unclear how the claimed method, as recited, is a "diagnostic" method. At best, the method steps appear to only provide identification of enriched fetal nucleated cells from a maternal whole blood sample; which if isolated, identified, and

imaged, can be used to provide genotypic and phenotypic characterization of the fetal nucleated erythrocytes to hence, provide a diagnostic capability. Please clarify.

Claim 2 is vague in reciting, "wherein the step of enriching *further comprises* the step of centrifugation" because it is unclear as to whether the step of centrifugation is a separate step that *further comprises* the enriching step, or does the centrifugation step intend to encompass the enriching step. If the former statement is true, then it is unclear what the "enriching step" is intended to encompass. If the latter statement is true, then claim 2 should recite, "wherein the step of enriching *comprises* the step of centrifugation".

Claim 4 is vague in reciting, "wherein the step of charge flow separation *further comprises* the step of imparting a buffer flow through a charge flow separator" because it is unclear as to whether the step of imparting a buffer flow through a charge flow separator is a separate step that *further comprises* the step of charge flow separation, or does the step of imparting a buffer flow through a charge flow separator intend to encompass the step of charge flow separation. If the former statement is true, then it is unclear what the "step of charge flow separation" is intended to encompass. If the latter statement is true, then claim 4 should recite, "wherein the step of charge flow separation *comprises* the step of imparting a buffer flow through a charge flow separator".

Claim 4 is objected to for the recitation of "an flow orientation vector". Claim 4 should properly recite, "a flow orientation vector".

Claim 5 is confusing in reciting "wherein the labeling step *further comprises* the step of binding a fetal cell-specific antibody" because it is unclear what structural and

functional cooperative relationship exists between the "fetal cell-specific antibody" in the instant claim and the "detectable label" recited in claim 1. It is also specifically unclear what is encompassed by "the detectable label" in claim 1 and how it relates to the "binding of fetal [labeled] cell-specific antibody" which is supposed to differentially encompass a separate label from that recited in claim 1; or does Applicant intend for the "detectable label" and "[labeled] fetal specific antibody" to be the same label. If Applicant intends the labeling step of claim 1, step b) to encompass a different detectable label, i.e. histochemical stain, from the "label" recited in the instant claim, i.e. monoclonal antibody label, then such should be clearly, distinctly, and differentially defined in the claimed invention.

Claim 5 is vague and indefinite in reciting, "fetal cell-specific antibody" because it is unclear as to whether the antibody is intended to be specific for any "fetal cell", including fetal mature (non-nucleate) erythrocytes. If so, it is unclear how the antibody should only bind fetal nucleated erythrocytes. It appears that claim 5 should properly recite, "fetal nucleated erythrocyte-specific antibody."

Claim 6 is vague and indefinite in reciting, "fetal cell-specific antibody" because it is unclear as to whether the antibody is intended to be specific for any "fetal cell", including fetal mature (non-nucleate) erythrocytes. If so, it is unclear how the antibody should only bind fetal nucleated erythrocytes. It appears that claim 6 should properly recite, "fetal nucleated erythrocyte-specific antibody."

Claim 6 is confusing in relation to claim 5 from which it depends because it is unclear how "a magnetic bead" for conjugation with the "fetal cell-specific antibody" is part of the labeling step. Please clarify.

Claim 7 is confusing in relation to claim 5 from which it depends in reciting, "where the binding step is followed by binding a fluorescent label" because it is unclear how "binding a fluorescent label" is effected within the labeling step. Does Applicant perhaps intend that the "fetal cell-specific antibody" is conjugated with a fluorescent label. It is specifically unclear what structural and functional cooperative relationship exists between this instant "fluorescent label", the "fetal cell-specific antibody" recited in claim 5, and the "detectable label" recited in claim 1, both from which the instant claim 7 depends. Does Applicant intend for all these "labels" to encompass one same label. If Applicant intends the labeling step of claim 1, step b) to encompass a different detectable label, i.e. histochemical stain, from the "label" recited in claim 5, i.e. monoclonal antibody label, and further from the "fluorescent label" in the instant claim, i.e. fluorescent FISH probe, then such should be clearly, distinctly, and differentially defined in the claimed invention. Please clarify.

In sum, if each of the "labeling elements" recited in claims 1, 5, and 7 are intended to be distinct elements, then all of claims 1, 5, and 7 should clearly, distinctly, and differentially define them as such, providing how one element directly or differentially relates from another. As an example, if Applicant intends the label in claim 1 to be a histochemical stain (DAPI for identifying DNA in nuclei), the label in claim 5 to be a fluorescent labeled monoclonal antibody (labeled fetal nucleated erythrocyte-

specific antibody for fetal nucleated red cell phenotyping), and the label in claim 7 to be a fluorescent in situ hybridization (FISH) probe (for karyotyping or genotyping X and Y chromosomes), it is suggested that each element be defined as such or equivalent language be used, to assist Applicant in obviating these indefiniteness issues. Although the claims are interpreted in light of the specification, limitations from the specification are not read into the claims. See *In re Van Geuns*, 988 F.2d 1181, 26 USPQ2d 1057 (Fed. Cir. 1993).

Claim 9 lacks antecedent basis in reciting, "the labeled features".

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> (e) the invention was described in (1) an application for patent, published under section 122(b), by another filed in the United States before the invention by the applicant for patent or (2) a patent granted on an application for patent by another filed in the United States before the invention by the applicant for patent, except that an international application filed under the treaty defined in section 351(a) shall have the effects for purposes of this subsection of an application filed in the United States only if the international application designated the United States and was published under Article 21(2) of such treaty in the English language.

Since claims 1-10 are so unclear and broad, as to the type, number, nature, and relationship of detectable labels used, the recitation of "detectable label", "[labeled] fetal cell-specific antibody", and "fluorescent label" within the context of the labeling steps in claims 1, 5, and 7, respectively, are read to encompass any one type of labeling, i.e. histochemical staining, fluorescent labeled antibody labeling, and fluorescent in situ hybridization probe labeling; alone, in combination, and in multiple versions thereof,

because unpatented claims are given the broadest interpretation consistent with the specification. Accordingly,

5. Claims 1, 2, 5, and 7-10 are rejected under 35 U.S.C. 102(e) as being anticipated by Ravkin (US Patent 6,169,816).

Ravkin discloses optical recognition systems for identifying fetal nucleated erythrocytes or from here on, fetal nRBCs, from maternal cells. The system performs image processing of fetal nRBCs labeled with different dyes, which are caused to reside on and define different portions of the fetal nRBCs such as the fetal hemoglobin in the cytoplasm and also the nuclei (see column 1, line 65 to column 2, line 11). Ravkin discloses obtaining maternal whole blood sample containing fetal nRBCs, enriching the population of fetal nRBCs in the sample, and labeling the fetal nRBCs in the enriched sample with a detectable label (labeled anti-fetal hemoglobin nRBC antibody, VECTOR BLUE substrate, DAPI, fluorescent labeled FISH probe) for positive identification and genetic analysis of the fetal nRBCs. Ravkin uses fluorescent labeled FISH probe to bind and identify DNA in the chromosomes of the fetal nRBCs. Ravkin also discloses use of multiple FISH probes or M-FISH which are labeled with different fluorescent dyes, i.e. different detectable labels, for identifying different portions of the fetal nRBC DNA (see column 2, line 60 to column 3, line 37). The enrichment procedure comprises centrifugation of the whole blood sample to remove major fractions of platelets and mature RBCs, then nRBCs are harvested from the interface between the white blood cells (WBCs) and the RBCs. The enriched portion is also subjected to selective lysis of

maternal RBCs and three layer density gradient to separate nRBCs from WBCs (see column 2, lines 38-56). Thereafter, fetal nRBCs are labeled with anti-fetal nRBC specific antibody, i.e. anti-fetal hemoglobin nRBC antibody or anti-HbF, then with secondary antibody indirectly conjugated to alkaline phosphatase for reaction with VECTOR BLUE substrate, which gives a blue precipitate on the cytoplasm of fetal nRBCs, and a DNA intercalating agent (DAPI) that gives the nuclei a fluorescent blue stain. The presence of these contrast labels, identifies, and determines the presence of fetal nRBCs (see column 3, line 64 to column 4, line 6 and column 5, lines 42-63). The nRBCs are detected by creating digitized images or fields from enriched samples on microscope slides containing labeled nRBCs, using a CCD (charge coupled device); the digitized images are subsequently sent to a computer for processing of the digitized images. The computer includes controlling motors for positionally controlling the microscope stage and the microscope slide containing the enriched fetal nRBCs sample (see column 4, lines 52-56, column 5, lines 12-25, and column 5, line 66 to column 6, line 10). Cytological features representative of fetal nRBCs are located and determined in a plurality of fields, then a plurality of positional coordinates are assigned therefor. Images are subsequently obtained, generated, and digitized from these cytological and morphological features (see column 7, lines 10-57). Ravkin discloses storing the digitized images and coordinates onto a web-based internet server (Internet's Word Wide Web) for remote access and manipulation of the stored digitized images. The digitized images may also be stored onto machine readable medium (hard drive, floppy) (see column 6, line 59 to column 7, line 5).

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

6. Claims 3 and 4 are rejected under 35 U.S.C. 103(a) as being unpatentable over Ravkin (US Patent 6,169,816) in view of Sammons et al. (US Patent 5,662,813).

Ravkin has been discussed supra. Ravkin differs from the instant invention in failing to teach that the enriching step further comprises charge flow separation.

Sammons et al. disclose a method for separation and enrichment of nucleated fetal erythrocytes or from here on, fetal nRBCs from maternal blood samples using charge flow separation. Sammons et al. teach obtaining maternal whole blood sample

containing fetal nRBCs, enriching the population of nRBCs by centrifugation and Ficoll gradient layering. The nucleated cell layer obtained from the maternal blood sample, is resuspended in buffer and then subjected to charge flow separation in a charge flow separator having a flow orientation vector which is opposite to applied electrical charge vector within the charge flow separator (buffer input flow rate of 0.270 ml/min/channel with a buffer output flow rate of 0.220 ml/min/channel, and a counterflow rate of 1.8 ml/min. in applied electric field of 250 V at 68-72 mA). After enrichment by charge flow separation, fetal nRBCs are collected and identified histologically with a detectable label. See Example 1. Sammons et al. provide that enrichment method may be operated stand alone or as a pre- or post- processing step in conjunction with the charge flow separation method (see Abstract).

It would have been obvious to one of ordinary skill in the art at the time of the instant invention to incorporate the teaching of Sammons in charge flow separation (CFS) for enriching rare fetal nRBCs, into the method of enriching and detecting fetal nRBCs as taught by Ravkin having incorporated therein, automated digitized imaging, processing, and storing of the labeled and identified fetal nRBCs, because Sammons specifically suggested application of the CFS method in conjunction or combination with any other cellular separation/enrichment methods. One of ordinary skill in the art at the time of the instant invention would have been motivated to combine the teaching of Sammons which incorporate charge flow separation, with other fetal nRBC enrichment methods such as that taught by Ravkin, wherein fetal nRBCs are enriched and detected

for further automated processing of digitized images because Sammons specifically provides that the CFS method successfully recovers fetal nRBCs from the peripheral circulation of pregnant women for histological identification, and since the recovered cells are viable, the nRBCs can be subjected to further enrichment methods such as cell culture.

7. Claim 6 is rejected under 35 U.S.C. 103(a) as being unpatentable over Ravkin (US Patent 6,169,816) in view of Alter (US Patent 5,580,724).

Ravkin has been discussed supra. Ravkin differs from the instant invention in failing to teach that the fetal cell-specific antibody is conjugated to a magnetic bead.

Alter discloses methods of differential expansion of fetal stem cells, including fetal nucleated erythrocytes or from here on, fetal nRBCs (erythroid progenitors), in whole blood obtained from maternal circulation for use in prenatal diagnosis purposes (see Abstract). In separation methods for separating fetal mononuclear cells, such as fetal nRBCs expressing CD71 (early erythroid cells which are nRBCs), Alter teaches incorporating magnetic beads into fetal nRBCs in order to separate and enrich the nRBCs from the maternal mononuclear cells, using magnetic activated cell sorting (MACS) (see column 8, lines 24-65). Selective enrichment is accomplished through binding or conjugation with antibodies specific for the nRBCs expressing CD71, i.e. anti-CD71 antibody conjugated to a magnetic bead (see also column 9, lines 10-34).

It would have been obvious to one of ordinary skill in the art at the time of the instant invention to incorporate the teaching of Alter in incorporating magnetic beads into nRBCs for enrichment processing by MACS, into the method of enriching and detecting fetal nRBCs as taught by Ravkin having incorporated therein, automated digitized imaging, processing, and storing of the labeled and identified fetal nRBCs, because Alter specifically suggested application of the MACS method in conjunction or combination with other cellular separation/enrichment methods. One of ordinary skill in the art at the time of the instant invention would have been motivated to combine the teaching of Alter which incorporates magnetic bead separation, with other fetal nRBC enrichment methods such as that taught by Ravkin, wherein fetal nRBCs are enriched and detected for further automated processing of digitized images, because Alter specifically taught that his method provides increased recovery yield of fetal nRBCs from the peripheral circulation of pregnant women, for use in prenatal diagnostic studies and purposes.

8. No claims are allowed.

Remarks

9. Prior art made of record are not relied upon but considered pertinent to the applicants' disclosure:

Zheng et al. (Prenatal diagnosis from maternal blood: simultaneous immunophenotyping and FISH of fetal nucleated erythrocytes isolated by negative magnetic cell sorting, Journal of Medical Genetics, 30 (12): 1051-1056 (December 1993)) teach simultaneous immunophenotyping and FISH of fetal nucleated erythrocytes isolated by negative magnetic cell sorting (see Abstract).

Mavrou et al. (Fetal Nucleated Erythrocytes (NRBCS) in Chorionic Villus sample supernatant fluids: an additional source of Fetal Material for Karyotype Confirmation, Prenatal Diagnosis 17 (7): 643-649 (1997)) teach a method for simultaneously detecting a phenotype and a genotype of a heme-containing cell such as fetal nRBCs in a sample. First, the fetal nRBC is contacted with a [first] fluorophore labeled mouse anti-fetal hemoglobin antibody (UCHγ) which binds an antigen in the nRBC in order to provide an immunophenotype of the cell. The cell is further contacted with a [second] fluorophore labeled DNA X- and Y- specific probe for fluorescence in situ hybridization (Two-colour FISH) analysis in order to provide a genotype (karyotype) of the cell (see Abstract and page 644, columns 1 and 2).

Pazouki et al. (A rapid combined immunocytochemical and fluorescence in situ hybridization method for the identification of human nucleated red blood cells, Acta Histochemica, 98 (1): 29-37 (January 1996)) teach a rapid combined immunocytochemical and fluorescence in situ hybridization method for the identification of human fetal nucleated red blood cells (see Abstract).

10. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Gailene R. Gabel whose telephone number is (571) 272-0820. The examiner can normally be reached on Monday, Tuesday, and Thursday, 7:00 AM to 4:30 PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Long V. Le can be reached on (571) 272-0823. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see.http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Gailene R. Gabel
Patent Examiner
Art Unit 1641
September 30, 2005

Notice of References Cited	Application/Control No. 10/009,085	Applicant(s)/Patent Under Reexamination SAMMONS ET AL.	
	Examiner Gailene R. Gabel	Art Unit 1641	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-6,169,816	01-2001	Ravkin, Ilya	382/128
	B	US-5,662,813	09-1997	Sammons et al.	210/806
	C	US-5,580,724	12-1996	Alter, Blanche P.	435/6
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Zheng et al., Prenatal diagnosis from maternal blood: simultaneous immunophenotyping and FISH of fetal nucleated erythrocytes isolated by negative magnetic cell sorting, Journal of Medical Genetics, 30 (12): 1051-1056 (December 1993).
	V	Mavrou et al., Fetal Nucleated Erythrocytes (NRBCS) in Chorionic Villus sample supernatant fluids: an additional source of Fetal Material for Karyotype Confirmation, Prenatal Diagnosis 17 (7): 643-649 (1997).
	W	Pazouki et al., A rapid combined immunocytochemical and fluorescence in situ hybridization method for the identification of human nucleated red blood cells, Acta Histochemica, 98 (1): 29-37 (January 1996).
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Prenatal diagnosis from maternal blood: simultaneous immunophenotyping and FISH of fetal nucleated erythrocytes isolated by negative magnetic cell sorting

Yun-ling Zheng, Nigel P Carter, Cathy M Price, Susan M Colman, Peter J Milton, Gerald A Hackett, Melvyn F Greaves, Malcolm A Ferguson-Smith

Abstract

Fetal nucleated cells in the maternal circulation constitute a potential source of cells for the non-invasive prenatal diagnosis of fetal genetic abnormalities. We have investigated the use of the Magnetic Activated Cell Sorter (MACS) for enriching fetal nucleated erythrocytes. Mouse monoclonal antibodies specific for CD45 and CD32 were used to deplete leucocytes from maternal blood using MACS sorting, thus enriching for fetal nucleated erythrocytes which do not express either of these antigens. However, significant maternal contamination was present even after MACS enrichment preventing the accurate analysis of fetal cells by interphase fluorescence in situ hybridisation (FISH). To overcome this problem, we used simultaneous immunophenotyping of cells with the mouse antifetal haemoglobin antibody, UCHγ, combined with FISH analysis using chromosome X and Y specific DNA probes. This approach enables selective FISH analysis of fetal cells within an excess of maternal cells. Furthermore, we have confirmed the potential of the method for clinical practice by a pilot prospective study of fetal sex in women referred for amniocentesis between 13 and 17 weeks of gestation.

(*J Med Genet* 1993;30:1051-6)

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Maternity, Gynaecology, and Genetic Services, Addenbrooke's NHS Trust, Rosie Maternity Hospital, Cambridge, UK.
P J Milton
G A Hackett

Correspondence to Dr Carter.

Received 12 July 1993. Accepted 13 September 1993.

There have been a number of reports showing the presence of nucleated cells of fetal origin in the maternal circulation during pregnancy. Although these cells are rare, they have generated great interest as a potential source of fetal cells for the non-invasive prenatal diagnosis of genetic abnormalities. Various fetal cell types (trophoblasts, erythrocytes, and leucocytes) cross the placenta and circulate within maternal blood.[1,2] The cell type which appears to offer the most potential for prenatal diagnosis is the fetal nucleated erythrocyte as fetal lymphocytes may persist from previous pregnancies and syncytiotrophoblast may not reflect the fetal genome. Fetal nucleated erythrocytes have been isolated from maternal blood during pregnancy using monoclonal antibodies and flow sorting[3-6] and have been detected as early as 11 weeks of gestational age.[3,6,7]

Analysis of fetal nucleated erythrocytes sorted from maternal blood generally has been restricted to the determination of fetal sex by use of the polymerase chain reaction (PCR) with Y specific primers. More recently, fluorescence in situ hybridisation (FISH) has been used for the detection of aneuploidies in flow sorted fetal cells.[7,8] Trisomy 21 was found in this way in blood samples taken from pregnant women either after or, in one case, before chorionic villus sampling (CVS). However, the main problem with this approach is that the majority of the nucleated cells analysed are of maternal origin even after FACS sorting. Therefore, the diagnosis of fetal aneuploidy, which relies on the detection of abnormal numbers of hybridisation signals, is insensitive in these samples owing to the high level of normal maternal cell contamination and the inability to distinguish fetal cells from maternal cells.

We have investigated the use of the Magnetic Activated Cell Sorter (MACS) for enriching fetal nucleated erythrocytes. Leucocyte common antigen, CD45, is present on the cell surface of all mature human leucocytes and CD32 is present on mature granulocytes. Neither of these antigens appears to be expressed on nucleated erythrocytes. We have used mouse monoclonal antibodies to these antigens and MACS sorting to enrich fetal nucleated erythrocytes from maternal leucocytes by negative selection. The isolated cells have then been analysed by using fluorescence immunophenotyping with an antibody specific for fetal haemoglobin (Hb F) simultaneously with FISH. In this way, fetal cells are identified by the presence of Hb F and sexed using X and Y specific probes hybridised to these nuclei. The ability to focus the diagnostic procedure exclusively on cells of fetal origin represents a substantial advance on previous techniques.

Materials and methods

BLOOD SAMPLES AND CELL PREPARATION

Peripheral blood (20 ml) was drawn into heparinised tubes from pregnant women before amniocentesis at between 13 and 17 weeks of gestational age. The blood was diluted 1:2 in phosphate buffered saline (PBS). Step gradients were prepared by pipetting 5 ml of a mixture of Histopaque-1119 and Histopaque-1077 (Sigma, Poole, UK) in the ratio of 3:1 (density of approximately 1109) into a 50 ml centrifugation tube and overlayering

with 5 ml of Histopaque-1077. The diluted blood was overlayered onto the Histopaque-1077 and then spun at 800 *g* for 30 minutes at room temperature. Cells at the plasma/Histopaque-1077 and Histopaque-1077/Histopaque-1109 interfaces and the complete Histopaque-1077 layer were removed into a fresh tube and washed once with PBS, once with PBS/BSA/azide (0·1% bovine serum albumin, 0·01% sodium azide in PBS), and then resuspended in PBS/BSA/azide at 10^6 cells per 100 µl. The cells were sorted within four to six hours of peripheral blood sampling. Newborn umbilical cord blood samples (obtained from the delivery unit at Rosie Maternity Hospital) and blood samples from normal male and female adults were prepared similarly and used to develop the methods and to act as controls.

ANTIBODY STAINING PROCEDURES

Isolated cells were incubated on ice for 25 minutes with 10 µl/10^6 cells of mouse IgG2a anti-CD45 (Serotec Ltd, Kidlington, UK) and 2 µl/10^6 cells of mouse IgG2a anti-CD32 (Serotec Ltd). The cells were washed in PBS/BSA/azide twice, resuspended at 10^7 cells per 80 µl of PBS/BSA/azide, and incubated at 6 to 12°C for 15 minutes with 20 µl/10^7 cells of magnetic microbeads conjugated to rat antimouse IgG2a antibody (Miltenyi Biotec GmbH). The stained cells were washed with PBS/BSA/azide once, resuspended in 2 ml of PBS/BSA/azide, and then immediately separated by MACS.

MAGNETIC CELL SORTING

Cells were separated by MACS (Miltenyi Biotec GmbH) using columns of type A1 or A2 according to the binding capacity of the column, as recommended by the manufacturer. The flow through the separation column is regulated by the gauge (G) of a disposable needle used at the outlet. Before use, columns were filled with 70% ethanol by injection from the bottom, washed with PBS/BSA (0·5% BSA in PBS), incubated at room temperature with PBS/BSA for 15 to 30 minutes to saturate non-specific binding sites, and then flushed with ice cold PBS/BSA/azide. The labelled cells were applied to the top of the column placed within the permanent magnet of the MACS separator (Miltenyi Biotec GmbH) and negative cells eluted by washing with three column volumes of PBS/BSA/azide at a flow speed of approximately 1·5 ml/min (22G needle). To increase the absolute recovery of negative cells, retained cells were processed a second time at a higher flow rate. The column was removed from the MACS separator and the bound cells flushed to the top of the steelwool matrix by injection of PBS/BSA/azide from the bottom. The column was replaced into the MACS separator and the cells passed through the column at a flow speed of approximately 3·5 ml/min (21G needle). The column was washed with 5 to 10 column volumes of PBS/BSA/azide and the two fractions of eluted cells pooled.

SLIDE PREPARATION AND IMMUNOPHENOTYPING

Mouse IgG1 antifetal haemoglobin antibody (UCHγ),[9] which binds specifically to fetal erythrocytes, was used to immunophenotype the isolated cells. The specificity of the UCHγ was tested on direct smears of cord blood and normal adult blood diluted 1 to 4 in PBS.

Preparations for microscopy were made using a cytocentrifuge (Shandon Southern, Runcorn, UK) from the cells isolated by MACS from maternal blood and from control samples. Cells from cord blood were used as positive controls and maternal cells before MACS separation were used as negative controls. The slides were air dried overnight, fixed in 2% formaldehyde in PBS at room temperature for 10 minutes, rinsed in Tris buffered saline (TBS) for two minutes twice, and washed in a TBS bath at room temperature for 10 minutes with magnetic stirring.

The staining procedures are modified from the method described by Cordell *et al*[10] and Price *et al*.[11] Slides were incubated in 10% normal goat serum (Sigma) in TBS at room temperature for 15 minutes and 30 µl of UCHγ culture supernatant, diluted 1:80 in TBS containing 10% normal goat serum, was added to each slide and incubated at room temperature for 60 minutes in a humidified chamber. After washing the slides in TBS as before, 30 µl of goat antimouse IgG1 alkaline phosphatase (Euro-Path Ltd, Bude, UK) diluted 1:60 was added to each slide and incubated at room temperature for 60 minutes in a humidified chamber (the antibody was diluted in TBS with 10% normal rat serum, incubated on ice for 30 minutes, and spun for 10 minutes in a microcentrifuge before use). The slides were then washed as above and two to three drops of alkaline phosphatase Vector Red substrate (Vector, Bretton, UK) were added to each slide. Colour development was monitored using a light microscope. Finally, the slides were washed in a TBS bath for 15 minutes, counterstained with haematoxylin, air dried, mounted in an aqueous mounting medium (Glycerol Gel, DAKO Ltd, High Wycombe, UK), and examined by light microscopy.

FLUORESCENCE IN SITU HYBRIDISATION AND DETECTION

After microscopic examination of the immunocytochemical staining, the coverslips were rinsed off by incubating in 2 × SSC/Tween-20 (0·05% Tween-20 in 2 × SSC, 1 × SSC = 0·15 mol/l sodium chloride and 0·15 mol/l sodium citrate, pH 7·0) at 50°C for 10 minutes and then the slides were washed in 2 × SSC/Tween-20 at 50°C for 15 minutes twice and dehydrated in ethanol series (70%, 70%, 90%, 90%). The immunophenotyped cells were first probed with GMGY10, a chromosome Y specific repeat probe,[12] and, if negative, reprobed with DXZ1, a chromosome X centromeric repeat probe.[13,14] Hybridisation of the cells was carried out as described previously[11] with the exception that cells and probe were denatured together for 10 minutes at 90°C. After

hybridisation overnight at 42°C, the coverslips were removed by rinsing in 2 × SSC and the slides were washed twice in 50% formamide/1 × SSC at 45°C for five minutes, washed once in 1 × SSC at 45°C for five minutes, and once in 0·5 × SSC at 45°C for five minutes. The slides were then treated with alternating layers of fluoresceinated avidin DCS (Vector) and biotinylated goat anti-avidin (Vector), both at 5 μg/ml concentration in 4 × SSC, 0·05% Tween-20 (4 × T) containing 10% human AB serum, for 30 minutes at 37°C until two layers of avidin were applied. After each incubation in avidin or anti-avidin, the slides were washed three times at 45°C in 4 × T for five minutes. After the last wash in 4 × T, the slides were rinsed in 2 × SSC, and then mounted in 2·0 μg/ml DAPI in Citifluor AF1 antifede (Citifluor Ltd, London, UK).

MICROSCOPY AND SIGNAL ANALYSIS

To evaluate the enrichment of the MACS sorting, artificial mixtures of antibody stained (positive) and unstained (negative) lymphocytes were used. Antibody labelled cells were fluorescence labelled by replacing the magnetic microsphere layer of the standard protocol with successive layers of biotinylated horse antimouse IgG (5 μl of 1·5 mg/ml per 10^6 cells, Vector), fluorescein isothiocyanate conjugated streptavidin (4 μl of 1 mg/ml per 10^6 cells, Vector), and biotinylated magnetic microbeads (5 μl per 10^8 cells, Becton Dickinson, Oxford, UK). The slides were coded and the number of fluorescence negative and positive cells counted using an epifluorescence microscope both before and after MACS sorting.

In addition, slides of cells from umbilical cord blood prepared before and after MACS sorting were stained using Rapi-Diff II (HD Supplies, Aylesbury, UK) and the total number of nucleated erythrocytes present counted.

Immunophenotyped cells after MACS sorting of maternal blood were scored using an ordinary light microscope with a 20 × objective. The position of UCHγ positive nucleated cells was recorded using a New England Finder (Graticules Ltd, Tonbridge, UK) and photographed. After FISH, the UCHγ positive cells were relocated and analysed using a confocal laser scanning microscope (MRC-600, Bio-Rad Microscience Ltd, Hemel Hempstead, UK). Analysis was carried out without knowledge of the fetal karyotype.

Results

The efficiency of the enrichment of rare cells by MACS was evaluated by using artificial mixtures of antibody stained and unstained cells (table 1). At the lowest tested frequency of one negative cell to 16·7 positive cells, MACS produced a purified negative fraction containing 11 negative cells to each positive cell. This represents an enrichment factor of 184.

The mouse anti-Hb F antibody, UCHγ, was used to detect fetal erythrocytes by immunocytochemical staining. Almost 100% of nucleated erythrocytes and mature red cells in newborn cord blood smears were stained with this antibody (figure A). We did not find any positive nucleated cells on the direct blood smear slides of three non-pregnant women (approximately 1/4 of the slide from each sample was scanned comprising at least 5×10^5 nucleated cells). However, in slides of Histopaque prepared cells from these three non-pregnant females, we found a small number of UCHγ positive nucleated cells (1·4 to 3 cells per million nucleated cells, 10 million cells scanned for each sample).

The efficiency of the hybridisation with GMGY10 and DXZ1 probes on immunostained cells was tested on cord blood and normal blood cells. The hybridisation efficiency in UCHγ negative cells was higher than in UCHγ stained positive cells (table 2). For example, hybridisation with GMGY10 showed single Y signals in 87% of UCHγ positive male cells (figure B) but 98% of UCHγ negative male cells.

The simultaneous immunophenotyping/FISH analysis was applied to MACS processed peripheral blood samples from six pregnant women in a pilot prospective study. Three blood samples from non-pregnant adult females were used as negative controls. After gradient centrifugation, between 30 and 50×10^6 cells were isolated from the mononuclear cell layer from 20 ml of blood. After MACS sorting, between 3 and 6×10^5 nucleated cells were collected from the magnetic negative fraction. UCHγ positive nucleated cells were detected in five out of six pregnant blood samples (table 3, figure C,E) and no UCHγ positive nucleated cells were detected in the three non-pregnant blood samples. In the samples from the pregnant women, FISH with GMGY10 and DXZ1 showed four male pregnancies and one female pregnancy (figure D,F). Fetal sex determined in this way was in exact agreement with the cytogenetic analysis of amniotic fluid samples (table 3).

Discussion

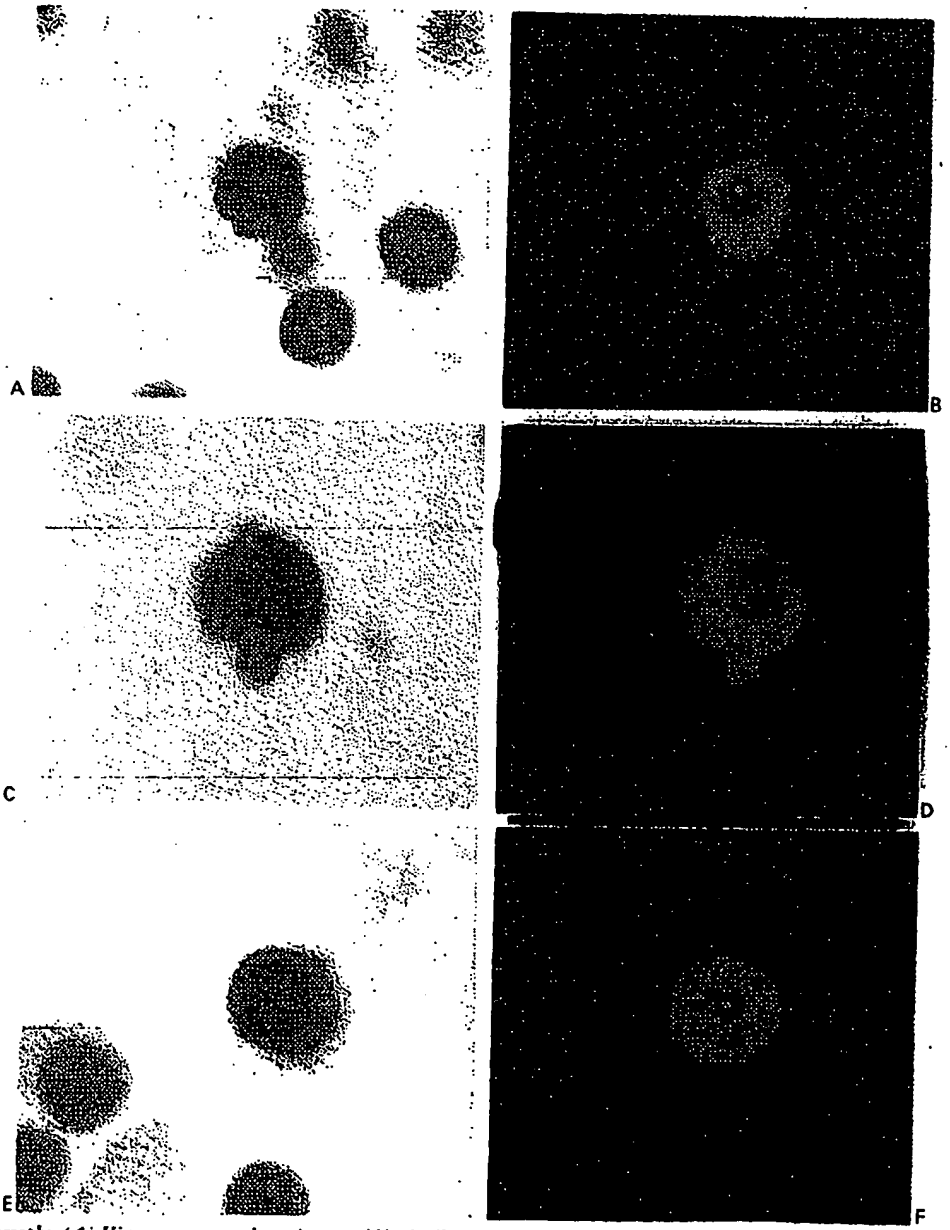
It has been shown by others that fetal nucleated erythrocytes can be isolated from maternal blood by FACS sorting using anti-transferrin receptor and anti-glycophorin-A antibodies.[5-8] However, this procedure is time consuming

Table 1 The efficiency of MACS separation using various ratios of labelled (+ve) and unlabelled (−ve) cells.

Before MACS sorting			After MACS sorting			
No of −ve/+ve cells	(Ratio)	Total No of −ve cells	No of −ve/+ve cells	(Ratio)	Total No of −ve cells	Enrichment (fold)
300/305	(1:1)	$1·0 \times 10^6$	632/36	(18:1)	$7·2 \times 10^5$	18
100/675	(1:7)	$3·3 \times 10^5$	618/72	(9:1)	$2·7 \times 10^5$	58
39/651	(1:17)	$2·0 \times 10^5$	627/57	(11:1)	$1·8 \times 10^5$	184

and the equipment is expensive. We have investigated the use of negative selection by MACS sorting to enrich fetal nucleated erythrocytes from maternal blood. MACS sorting is a fast and inexpensive method compared to FACS and we have found that 20 ml of prepared blood can be sorted in 10 minutes. The quality of MACS sorting depends not only on the immunomagnetic reaction, but also on the elution speed and volume. In this study, we used a minimal antibody concentration with a high sorting speed and large washing volumes to increase the absolute recovery rate of nucleated erythrocytes. Unfortunately, this strategy generates a high level of maternal nucleated cell contamination.

To date, there have been few reports of aneuploidy analysis by FISH of fetal cells from the maternal blood.[156] Fetal cells circulating in maternal blood are rare and, as described above, the majority of nucleated cells sorted are of maternal origin. This is true even after FACS sorting.[2156] The identification of fetal aneuploidy by interphase FISH relies on the detection of abnormal numbers of hybridisation signals in cell nuclei. Diagnosis using these sorted samples is inevitably insensitive and unreliable owing to the high level of



Micrographs. (A) Histopaque prepared new born cord blood cell smear showing a nucleated erythrocyte positive for Hb F by alkaline phosphatase-Vector Red (AP-VR) staining. (B) A confocal image of a male nucleated erythrocyte from cord blood after anti-Hb F/AP-VR staining (red fluorescence) and FISH with GMG Y10 probe (green fluorescence). (C) An anti-Hb F stained positive cell isolated from one of the maternal blood samples. (D) A confocal image of the same cell as in (C) after FISH with GMG Y10 probe showing a male genotype with one Y signal. (E) An anti-Hb F stained positive cell isolated from a second maternal blood sample. (F) A confocal image of the same cell as in (E) after FISH with DXZ1 probe showing a female genotype with two X signals.

Table 2 Hybridisation signal distribution in UCHγ positive and negative immunostained cells.

UCHγ staining	No of cells (%)						
	GMGY10 (male cells)			DXZ1 (female cells)			
	0	1	2	0	1	2	3
Positive	37 (12·4)	259 (86·9)	2 (0·6)	60 (12·6)	91 (19·1)	325 (68·3)	0 (0)
Negative	7 (1·5)	457 (97·7)	4 (0·9)	16 (3·4)	34 (7·3)	415 (88·5)	4 (0·9)

maternal cell contamination and the inability to distinguish fetal from maternal cells. We have shown in this study that the combination of immunophenotyping with mouse monoclonal anti-Hb F antibody and FISH analysis allows the identification of fetal nucleated red blood cells within the excess of maternal cells and enables chromosome copy number to be determined for fetal cells alone. This combination of staining technologies represents a substantial advance on previous FISH analyses of fetal cells sorted from maternal blood.

The combination of FISH and immunophenotyping has been used by others for the simultaneous analysis of phenotype and genotype in both mitotic and interphase cells.[11 16 17] Price *et al*[11] exploited the observation that the alkaline phosphatase-Fast Red reaction produces a bright red fluorescence that is visible by epifluorescence microscopy using both fluorescein and rhodamine filters. Fast Red immunofluorescence and FITC FISH signals can be visualised simultaneously in the same cell. We used a mouse monoclonal anti-fetal haemoglobin antibody (UCHγ), which specifically binds to fetal erythrocytes, to immunophenotype the MACS processed cells. We used Vector Red as a substrate as it produces red fluorescence similar to but brighter than Fast Red.

Interestingly, we observed that UCHγ also binds to a small number of nucleated cells (1·4 to 3 cells per million nucleated cells) in Histopaque prepared cells from three non-pregnant women. We also noted that these UCHγ positive nucleated cells were morphologically similar to lymphocytes rather than to nucleated erythrocytes. After MACS sorting, we were unable to find these UCHγ positive cells in the magnetic negative fraction sorted from the same three blood samples. The lineage of these cells is unknown but they represent a potential problem for the analysis of true fetal cells isolated from maternal blood and identified using UCHγ. Indeed, in the male pregnancy of case 11, one UCHγ positive cell obtained by MACS enrichment showed two copies of the X chromosome and no Y signal (table 3), which suggests that this cell was of maternal origin. This one maternal UCHγ positive cell did not affect the determination of fetal sex as the majority of the other UCHγ positive cells showed Y hybridisation signals. It is clear that the precise nature and origin of these UCHγ positive maternal cells should be studied further.

In four other samples from pregnant women, fetal sex determined by X and Y probe hybridisation in the UCHγ positive nucleated cells obtained by MACS enrichment was also in exact agreement with fetal sex determined by fetal chromosome analysis (table 3, figure). In the remaining one case studied, no UCHγ positive cells were detected. Possible explanations for the lack of UCHγ positive cells in this case include failure of the isolation process or the absence of fetal cells from the maternal blood. It is possible that ABO blood group differences between the mother and fetus could result in the rapid clearance of fetal cells from the maternal circulation.[18]

Currently, FISH used with interphase nuclei is not reliable for the diagnosis of all trisomies.[10 21] Problems of this technique include non-specific signals and incomplete hybridisation. It is also possible for signals to overlap in the two dimensional image of the three dimensional nucleus giving the incorrect number of signals. In other studies, the expected number of domains determined by interphase analysis with chromosome specific repeat probes varied between 67% and 98% of cells counted.[23-24] Clearly, the hybridisation efficiency is a major factor influencing the accuracy of the diagnosis. However, it has been shown that for autosome aneuploidy analysis, a signal distribution of greater than 40% of cells showing the expected number of signals is required to give a reliable diagnosis of trisomy.[22]

In this study, hybridisation with DXZ1 and GMGY10 probes in UCHγ positive cells showed the expected number of signals in 68% and 87% of cells counted, respectively. As the fetal cells isolated from 20 ml of maternal blood are so few (table 3), the probability of incorrect diagnosis owing to inefficient hybridisation is of concern. If we assume that more than 50% of cells must show the expected number of signals to generate a correct diagnosis, we can calculate the probability of an incorrect diagnosis using the binomial distribution.[25] Where we have only four cells to analyse with DXZ1 which produces two signals in 68% of UCHγ positive normal female

Table 3 Details of six blood samples from pregnant women studied by MACS/UCHγ staining/FISH.

Case	No of Hb F+ cells after MACS sorting	FISH Y10 signals		FISH DXZ1 signals			Gestation (weeks)	Sex by MACS/FISH	Karyotype by CAAF*
		0	1	0	1	2			
8	2	0	2	Not done			14	M	46,XY
9	3	3	0	0	1	2	17	F	46,XX
10†	11	3	8	3	0	0	13	M	46,XY
11‡	6	2	3	0	1	1	13	M	46,XY
12	3	0	3	Not done			13	M	46,XY
13	0	Not done		Not done			13	Failed	46,XY

* CAAF = cytogenetic analysis of amniotic fluid.
† Three Hb F positive cells, which were both Y and X hybridisation negative, were in the middle of a cell clump and overlapped by other cells.
‡ One of the Hb F positive cells was covered by other cells and could not be analysed by FISH.

fetal cells (table 2), the probability of a misdiagnosis (0, 1, or 2 cells showing the expected number of signals) is 0·36. However, if the hybridisation efficiency is 95%, the probability of incorrect diagnosis with four cells is greatly reduced ($p = 0·01$). If 10 cells are available for analysis by a probe with a hybridisation efficiency of 95%, the probability of incorrect diagnosis is very low ($p = 6 \times 10^{-5}$). Therefore, only a slightly more efficient MACS sorting technique, an increased volume of maternal blood, or more efficiently hybridising probes are needed for reliable routine diagnosis using the methods presented in this paper.

In conclusion, the MACS is a faster, less expensive technique than the FACS and allows a large volume of blood to be separated in a short period of time. While at present few fetal cells are recovered and maternal cell contamination is too great by both methods to allow accurate direct analysis by FISH, immunophenotyping with antibodies specific to cells of fetal origin enables selective FISH analysis of fetal cells within the excess of maternal cells. An important feature of this approach is that diagnostic results are available within three days after peripheral blood sampling. The application of multicolour FISH to these immunophenotyped cells would allow the visualisation of several probes simultaneously[26 27] and provide the potential for the detection of the common fetal aneuploidies directly from maternal peripheral blood samples. The methods presented in this paper represent a substantial advance on previous techniques used for analysis of fetal cells isolated from maternal blood and show that non-invasive prenatal diagnosis from maternal blood has the potential to become a practical routine procedure.

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FETAL NUCLEATED ERYTHROCYTES (NRBCS) IN CHORIONIC VILLUS SAMPLE SUPERNATANT FLUIDS: AN ADDITIONAL SOURCE OF FETAL MATERIAL FOR KARYOTYPE CONFIRMATION

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SUMMARY

Fetal erythrocytes leak from the fetal capillaries at the time of chorionic villus removal. The purpose of this study was to determine if fetal nucleated erythrocytes (NRBCs) could be isolated from the chorionic villus sampling (CVS) supernatant fluid and used as an additional source of fetal material in order to confirm the fetal karyotype in cases of CVS mosaicism. One hundred CVS supernatant fluids were studied by simultaneous immunophenotyping, using a mouse antifetal haemoglobin antibody, UCH*y*, combined with fluorescent *in situ* hybridization (FISH) analysis using X- and Y-specific DNA probes. A chromosome 18 probe was also used in the case of a known male fetus with trisomy 18. Fetal haemoglobin (HbF)-positive cells were identified in 73 supernatant fluids and HbF-positive nucleated cells were present in 60 samples. The number of cells-detected per sample showed great variation among the individual samples. FISH analysis was performed in 41 cases. FISH prediction of the fetal gender was concordant with the CVS karyotype in all cases, and the fetal trisomy 18 was correctly verified. In five cases in which Y sequences were detected, a small number of HbF-positive cells with two X signals were also identified; interestingly, in three of the five cases, the mother was a β-thalassaemia carrier. This technique can be used as a quick and accurate method for the immediate verification of CVS results in cases of mosaicism, thus avoiding second-trimester amniocentesis. © 1997 by John Wiley & Sons, Ltd.

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KEY WORDS: CVS supernatant fluid; fetal haemoglobin; CVS mosaicism; prenatal diagnosis

INTRODUCTION

Prenatal diagnosis for chromosomal anomalies, using chorionic villus sampling (CVS), has been widely accepted as an alternative to amniocentesis. An area of concern, however, is the existence of mosaicism and its effect on the subsequent interpretation of test results. CVS mosaicism, detected in approximately 1 per cent of cases (Schreck *et al.*, 1990; Ledbetter *et al.*, 1992; Bianchi *et al.*, 1993), is usually due to a discordance in the karyotype between the fetus and the placenta and in the majority of cases, the abnormal cell line is confined to the placenta (Kalousek and Dill, 1983; Artan *et al.*, 1995). In practice, amniocentesis during the second trimester of pregnancy is performed in order to clarify the fetal genotype, but this incurs a delay in the reporting of results

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due to time-consuming cell cultures. That is why it would be helpful if the karyotype of the fetus could be confirmed at the time of CVS.

The erythrocyte content and haemoglobin concentration increase with fetal development. Around the 12th week of pregnancy, the nucleated red cells may number up to 50 000 per mm (Thomas and Yoffey, 1962). Fetal erythrocytes leaking from the fetal capillaries at the time of villus removal may constitute a potential additional source of fetal genetic material. The aim of this study was to determine if fetal nucleated red blood cells (NRBCs) could be identified in the CVS supernatant, using a monoclonal antibody against the γ chain of fetal haemoglobin and chromosome-specific probes.

MATERIALS AND METHODS

The study sample was composed of 100 CVS supernatant fluids. Sampling was carried out at 10–12 weeks of gestation by conventional techniques, mainly transabdominally. Villi were collected in RPMI-1640 transfer medium (GIBCO BRL).

For the first 26 samples, immunophenotyping was performed in a qualitative manner, looking for the presence or absence of fetal haemoglobin-positive NRBCs in the CVS supernatant fluids. When we were certain that we could identify NRBCs by this method, we proceeded in the next 74 samples to count and subsequently analyse the cells by fluorescent *in situ* hybridization (FISH), using X- and Y-specific probes for identification of the fetal gender. The CVS supernatant fluids were obtained from Greek patients undergoing prenatal diagnosis for the following reasons: advanced maternal age ($N=29$), recurrence risk for chromosomal aneuploidy ($N=7$), abnormal ultrasound findings ($N=1$), and recurrence risk for monogenic disorders ($N=35$). In the last group, 13 samples were from pregnancies tested prenatally for thalassaemia. Two cases were performed for unknown reasons. One of the cases performed for advanced maternal age was a known male fetus with trisomy 18.

Methods

After setting up the CVS cultures, the transfer media containing residual fetal blood cells were centrifuged at 1200 rpm for 5 min and smears were prepared from the pellet on one or two slides. The preparations were air-dried overnight and stored at −20°C.

For immunophenotyping of the isolated cells, UCHγ, a mouse monoclonal IgG1 antifetal haemoglobin antibody binding specifically to the γ chain of fetal erythrocytes, was used.

For FISH, commercially available biotinylated DXZ1, digoxigenin-labelled DYZ3, and biotin-labelled D18Z1 probes were used (Oncor, Inc.).

Immunophenotyping

Slides were fixed for 10 min in 2 per cent formaldehyde in phosphate-buffered saline (PBS) at room temperature, rinsed in Tris-buffered saline (TBS) for 2 min twice, and washed for 10 min in TBS.

Staining procedures were slightly modified from the method described by Zheng *et al.* (1993). 20 μl of 10 per cent normal goat serum in TBS was added to each slide and the preparations were incubated for 15 min in a humidified chamber. 100 μl of UCHγ was added to each slide and incubated for 60 min at room temperature in a humidified chamber. After washes in TBS, 30 μl of goat anti-mouse IgG1 alkaline phosphatase diluted 1:50 in TBS was added to each slide and the preparations were incubated in a humidified chamber for 60 min. For colour development, the slides were covered with alkaline phosphatase, Vector Blue substrate and incubated at room temperature for 10 min. The preparations were washed again in TBS, counterstained with the fluorescent dye DAPI (4,6-diamino-2-phenylindole), and mounted in glycerol gel.

The slides were carefully screened, using fluorescent and light microscopy, and all the UCHγ-positive cells, hereafter referred to as HbF-positive cells, were counted. The red blood cells that were HbF-positive and nucleated were counted separately and their positions on the slide were recorded.

Fluorescent in situ *hybridization (FISH)*

Immunophenotyping only was performed in the first 26 samples. In the remaining samples, FISH analysis was performed on the identified HbF-positive NRBCs in cases where more than 4–5 cells were recorded on the slide.

Two-colour FISH was applied in 27 samples, using a modification of the procedure described in Zheng *et al.* (1993). After microscope examination,

the coverslips were rinsed off by incubating the slides for 5 min in 2 × SSC at 42°C. The slides were washed twice in Tween-20/2 × SCC for 15 min at 42°C, washed once in 2 × SSC for 15 min at 42°C, and dehydrated through an ethanol series. Two-colour FISH was performed using biotinylated DXZ1 and digoxigenin-labelled DYZ3 probes. For detection of the chromosome 18 copy number, in one case of a known trisomy 18, biotin-labelled D18Z1 was used.

Hybridization mixture, consisting of 50–100 ng of biotinylated DNA probe (DXZ1), 1 μl of human cot-1 DNA, 15 μl of hybridization buffer, and 50–100 ng of digoxigenin-labelled DNA probe (DYZ3), was placed on each slide and sealed under a coverslip. The cells and probes were denatured together by heating for 10 min at 85°C and hybridization was carried out overnight at 42°C. After hybridization, the slides were washed twice in 50 per cent formamide diluted in 2 × SSC for 5 min at 42°C, washed twice again in 2 × SSC at 42°C for 5 min, and incubated in 4XTNFM (4 × SSC, 0·05 per cent Tween-20, 5 per cent non-fat milk) at 37°C for 20 min.

For signal detection, when two-colour FISH was performed, the slides were first incubated with a layer of FITC-labelled avidin, diluted in 4XTNFM, for 30 min at 37°C in a humidified chamber. Next they were incubated for 60 min with mouse anti-Dig and biotinylated goat anti-avidin at 37°C and finally they were incubated with a mixture of FITC-labelled avidin and Cy3-labelled goat anti-mouse for 60 min at 37°C. Between incubations there were three 5-min washes at 42°C in 4XTNFM. The slides were finally washed twice in 2 × SSC, counterstained with DAPI, mounted in Citifluor, sealed, and examined.

In 14 samples, X and Y probes were applied simultaneously in two different regions of the same slide and *in situ* hybridization was carried out according to the procedure described by Zheng *et al.* (1992). For signal detection, the slides were treated with alternating layers of FITC-labelled avidin and biotinylated goat anti-avidin.

The HbF-positive nucleated cells were relocated and analysed by epifluorescence microscopy, using DAPI, fluorescein, and rhodamine filters.

RESULTS

A total of 100 CVS supenatant fluids were studied. Of these, 27 samples showed no cells or contained only cell debris. Of the 73 samples with cells present, HbF-positive cells were identified in all cases and HbF-positive nucleated cells were identified in 60 samples. The total number of HbF-positive cells, nucleated and non-nucleated, detected per sample showed great variation among the individual samples. The mean number of HbF-positive cells per sample was 351 (range 11–1160). The mean number of nucleated HbF-positive red blood cells was 23 (range 1–355). Forty-eight samples (73 per cent) had more than five nucleated red blood cells. Details on some of these findings are presented in Table I.

FISH analysis was performed in 41 cases and bright FISH signals were seen in 37 samples. The mean number of cells that showed FISH signals per sample was 23 (range 6–34). Hybridization efficiency was 74 per cent for the X and 65 per cent for the Y probe, respectively. FISH analysis showed that 19 fetuses were females and 18 males. In all cases, the FISH prediction of the fetal gender was concordant with the CVS karyotype (Figs 1 and 2). In five of the 18 CVS samples in which Y sequences were detected, a small number of maternal NRBCs showing two copies of the X chromosome were also identified (Table I). In one case of a known male fetus with trisomy 18, 24 HbF-positive nucleated cells were identified by immunophenotyping. Two-colour FISH with chromosome 18- and Y-specific probes demonstrated that 16 cells showed three 18 signals. Twenty-three cells showed a Y signal (Table II and Fig. 3).

DISCUSSION

Immunophenotyping using an anti-γ-chain monoclonal antibody revealed that 73 per cent of CVS supernatant fluids contained varying numbers of fetal nucleated erythrocytes. In 48 per cent of the cases more than five NRBCs were identified, thus permitting reliable interphase FISH analysis. The number of fetal cells identified in the CVS supernatant fluids varied. In certain samples, the NRBCs were so numerous that we wondered about the long-term effect of fetal blood loss early in gestation. To our knowledge, all the babies born up to date were normal. This is in agreement with previous reports (Smidt-Jensen *et al.*, 1986; Los *et al.*, 1989) that there seems to be no correlation between exsanguination of the fetus due to voluminous

Table I—Results of FISH and HbF staining on CVS supernatant fluids

Sample No.	Indication	No. of HbF-positive cells	No. of nucleated HbF-positive cells	Sex by FISH	CVS karyotype
1	Haemophilia	102	39	M	46,XY
2	Previous chrom. abnormality	180	54	F	46,XX
3	AMA	197	59	F	46,XX
4	SMA	180	32	M/F, 6%	46,XY
5	AMA	480	72	F	46,XX
6	Thalassaemia	499	34	F	46,XX
7	AMA	180	24	M, trisomy 18	47,XY,+18
8	Thalassaemia	380	17	M/F, 29%	46,XY
9	BMD	480	25	F	46,XX
10	AMA	324	20	M	46,XY
11	AMA+congen. adren. hyperplasia	70	12	M	46,XY
12	AMA	433	30	M/F, 20%	46,XY
13	Cystic fibrosis	152	10	F	46,XX
14	Previous Turner syndrome	24	7	F	46,XX
15	DMD	113	6	F	46,XX
16	AMA	175	19	M	46,XY
17	AMA+thalassaemia	345	11	M	46,XY
18	AMA	325	12	F	46,XX
19	AMA	680	355	F	46,XX
20	Haemophilia	151	18	M	46,XY
21	AMA+previous Down syndrome	200	12	M	46,XY
22	?	185	20	M	46,XY
23	AMA+thalassaemia	162	25	F	46,XX
24	Cystic fibrosis	157	32	M	46,XY
25	AMA	87	47	M	46,XY
26	AMA	238	51	M	46,XY
27	Previous child with trisomy 18	412	47	F	46,XX
28	Thalassaemia	741	148	F	46,XX
29	DMD	131	18	F	46,XX
30	Haemophilia	155	55	M	46,XY
31	Thalassaemia+AMA	326	85	M/F, 15%	46,XY
32	Thalassaemia	228	32	M/F, 16%	46,XY
33	AMA	520	93	F	46,XX
34	AMA	267	72	F	46,XX
35	Haemophilia	18	6	F	46,XX
36	AMA	70	10	F	46,XX
37	AMA	52	8	F	46,XX

AMA=advanced maternal age; SMA=spinal muscular atrophy; DMD=Duchenne muscular dystrophy.

feto-maternal transfusion at the time of CVS and subsequent fetal development.

Our study demonstrates that interphase FISH can be successfully performed on the immunophenotyped cells for the determination of fetal gender. The verification of trisomy 18 from the isolated fetal cells suggests that the use of this method may help, in cases of CVS mosaicism, to distinguish between fetal mosaicism and confined placental mosaicism. The hybridization efficiency could be improved and it is possible that with experience the FISH results will also improve.

In five CVS supernatant fluids in which the fetus was shown correctly by FISH to be male, nucleated HbF-positive cells showing two X signals were also identified in 6–29 per cent of total cells. The presence of a small number of female HbF-positive cells may be explained as a result of

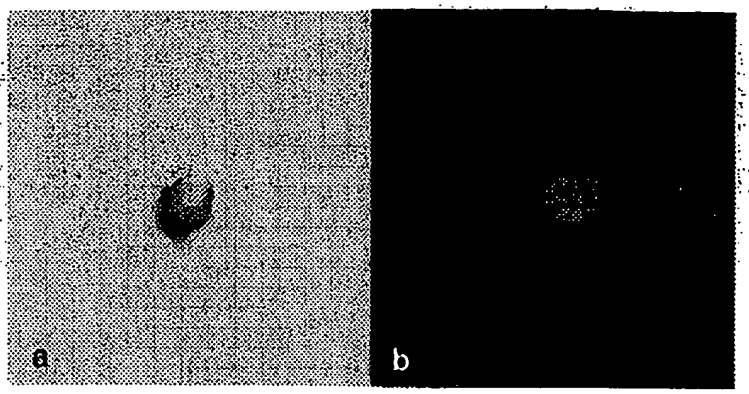

Fig. 1.—(a) Nucleated HbF-positive cell stained with alkaline phosphatase-Vector blue. (b) Same cell after FISH with the biotinylated DXZ1 probe, showing two X signals

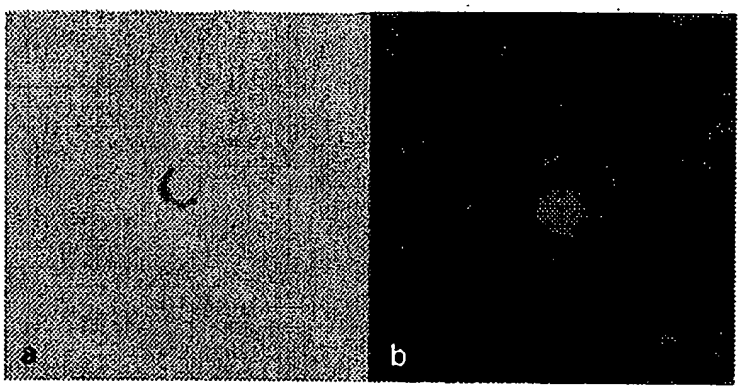

Fig. 2.—(a) Nucleated HbF-positive cell stained with alkaline phosphatase-Vector blue. (b) Same cell after FISH with the biotinylated DXZ1 probe and digoxigenin-labelled DYZ3 probe, showing one X (green fluorescence) and one Y (red fluorescence) signal

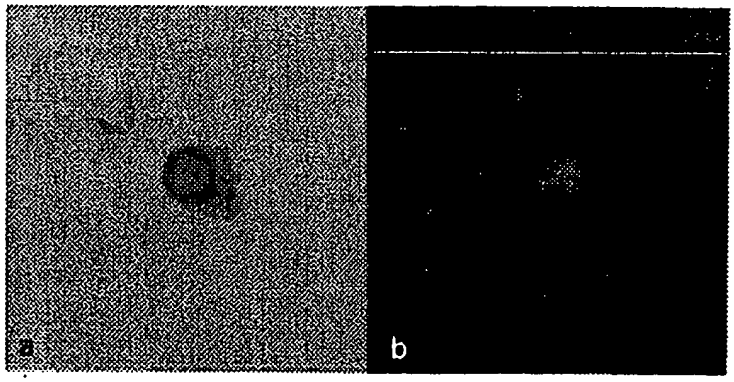

Fig. 3.—HbF-positive nucleated cell isolated from the CVS sample of a male fetus with trisomy 18. (a) Cell stained with alkaline phosphatase–Vector blue. (b) Same cell after FISH showing three chromosome 18 signals (green fluorescence) and one Y signal (red fluorescence)

Table II.—Distribution of signals in HbF-positive cells isolated from the supernatant of a CVS (fetus with trisomy 18)

	FISH D18Z1 signals					FISH DYZ3 signals		
No. of signals	0	1	2	3	4	0	1	2
No. of cells	0	1	6	16	1	1	22	1

a twin pregnancy, in which the female embryo was lost early in gestation. Another possibility is that we are looking at an artifact in which a maternal cell is overlapping a HbF-positive fetal cell. The most likely explanation, however, is that the cells showing two X signals were maternal nucleated red blood cells. Their number was too small to affect the correct determination of sex, but caution must be taken about the possibility of identifying maternal NRBCs by this method. It is known that materno-fetal transfusion may occur during CVS (Los *et al.*, 1996) and that pregnancy *per se* induces the appearance of maternal NRBCs in the maternal circulation (Slunga-Tallberg *et al.*, 1995). In cases in which the mother is anaemic, one can assume that under the stress of pregnancy an increased number of NRBCs will be released in her circulation. As has already been stated, 13 of the CVS samples included in this study came from pregnancies at risk for thalassaemia. We were able to isolate NRBCs in seven out of the 13 cases and correctly identified three female and four male fetuses. The mean number of NRBCs found in these samples was twice as high as the mean number of NRBCs found per sample in the study (50 as opposed to 23). In three of the four cases in which the fetus was a male, we also identified in varying degrees NRBCs showing two X signals. A possible explanation may be that in these cases the mothers, who were obligate carriers of the thalassaemia trait, were under the stress of pregnancy, producing nucleated red blood cells themselves. When the fetuses were females, we could not verify this observation, as we had no way of discriminating fetal from maternal NRBCs. Use of monoclonal antibodies that recognize antigens unique to fetal cells, such as anti-ζ- or anti-ε-chain antibodies, may allow the distinction between fetal and maternal NRBCs.

The results from this study demonstrate that isolation of NRBCs from CVS supernatant fluids is a technique that can be used as a possible quick and accurate method for the verification of CVS results in cases of mosaicism, thus avoiding the need for second-trimester amniocentesis. Its application does not require the use of valuable CVS material. FISH confirmation of aneuploidy can help to determine the chromosomal status of the fetus in cases of failed culture, or parental request for verification of the prenatal diagnosis findings. It may be useful, therefore, for prenatal diagnostic laboratories to routinely prepare slides from the supernatant at the time of CVS culture, store, and retrieve them if the need arises.

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A rapid combined immunocytochemical and fluorescence in situ hybridisation method for the identification of human fetal nucleated red blood cells

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Summary

Fetal nucleated red blood cells are found in the maternal circulation during pregnancy. If a simple routine method of detection of these cells was developed, it could be used as the basis of non-invasive prenatal diagnosis of fetal genetic disorders. Fetal male and adult female blood were mixed to mimic maternal blood in pregnancy and used to establish a simple technique to unequivocally detect fetal nucleated red blood cells. These were identified by combined immunocytochemistry using a human fetal haemoglobin antibody and a rapid and simple-to-use fluorescence in situ hybridisation method using X and Y chromosome probes. Initial studies using the alkaline phosphatase anti-alkaline phosphatase technique as the first procedure showed that the stain was unstable and unsuitable for in situ hybridisation. An immunoperoxidase technique was found to produce a stable stain resistant to harsh fixation steps required in subsequent in situ hybridisation. This enabled the simultaneous visualisation of immunopositivity and in situ hybridisation signals on the same cell with neither procedure affecting the other's signal quality.

We are currently using this procedure to detect a range of endoplasmic reticulum proteins in fetal nucleated red blood cells from maternal blood in an attempt to diagnose disorders of liver protein expression in early pregnancy.

Key words: peroxidase anti-peroxidase – immunocytochemistry – fluorescence in situ hybridisation – nucleated red blood cells – prenatal diagnosis

Introduction

Several investigators have in recent years reported the presence of fetal nucleated red blood cells (FNRBCs) in maternal peripheral blood (e.g. Holzgreve et al., 1992). The abundance of these cells in the maternal circulation remains controversial, with values ranging from 1 in 100,000,000 to 1 in 4,000 fetal to maternal nucleated cells (Adinolfi

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et al., 1989; Schwinger et al., 1989; Bianchi et al., 1990; Ganshirt-Ahlert et al., 1990; Nakagome et al., 1991; Price et al., 1991; Kao et al., 1992; Hamada et al., 1993). These FNRBCs have been the focus of much recent attention as they could provide a means of non-invasive prenatal diagnosis of fetal genetic disorders (e.g. Bianchi, 1994; Davies et al., 1994; Elias et al., 1994; Ganshirt et al., 1994) if a simple method of detection were developed. Fetal nucleated red cells are first enriched from maternal blood by density gradient centrifugation (Bhat et al., 1993), fluorescence activated cell sorting (Bianchi et al., 1993), magnetic activated cell sorting (Ganshirt-Ahlert et al., 1993) or some combination of these methodologies. Enriched cell fractions are then analysed for the presence of abnormalities by either the polymerase chain reaction (PCR) (Wachtel et al., 1991) or by fluorescence in situ hybridisation (FISH) (Davies et al., 1994).

However, to be able to confidently diagnose disorders using fetal nucleated red blood cells, it is important to first characterise them unequivocally, discriminating between fetal and any circulating maternal nucleated red blood cells. A monoclonal antibody raised against fetal haemoglobin, UCHγ (Allen et al., 1987) has recently been used for immunophenotyping of FNRBCs (Zheng et al., 1993) and it is the only available specific FNRBCs immunoprobe. However, immunophenotyping alone will not unequivocally distinguish between fetal and maternal nucleated red blood cells because of the possibility of expression of low levels of fetal hemoglobin in adults which is greatly exaggerated in maternal hemoglobinopathies (Huisman, 1993). We have therefore combined immunophenotyping using UCHγ with FISH using X and Y chromosome probes to identify fetal nucleated red blood cells. Although combined immunocytochemistry and FISH have been used for simultaneous immunophenotypic and genotypic analysis of cells, most investigators have so far used alkaline phosphatase anti-alkaline phosphatase (APAAP) immunocytochemistry coupled to FISH (Kibbelaar et al., 1992; Price et al., 1992; Weber-Matthiesen et al., 1992; Zheng et al., 1993). For example, Price et al., 1992) applied simultaneous APAAP and FISH to lineage analysis in patients with the myeloproliferative disease polycythemia vera using trisomy of chromosome 8 as a marker of the abnormal clone. They made use of the fluorescence properties of the APAAP reaction product to simultaneously visualise both immunophenotype and genotype by fluorescence microscopy.

In this paper we describe a simple routine method combining the immunoperoxidase (PAP) reaction with FISH. The immunocytochemical detection method produces a permanent brown precipitate which is preserved throughout the harsh fixation steps we found were necessary for FISH. Contrary to previous reports the diaminobenzidine (DAB) reaction product does not affect the accessibility of nuclei to the FISH probes (van den Brink et al., 1990). Moreover, the DAB precipitate does not interfere with the fluorescent signals obtained after FISH.

Materials and methods

Tissue samples. Human embryonic tissue was obtained within 1 h following termination of pregnancies of duration < 56 days amenorrhoea using Mifepristone (Roussel, Uxbridge, U.K.) and Gemeprost vaginal pessaries (May and Baker, Dagenham, U.K). Stages of embryonic development and the approximate postovulatory days of age were made according to established morphological criteria (O'Rahilly and Muller, 1987). Eight embryos (postovulatory age 32–56 days) were fixed in 10% buffered formaldehyde for 5 days and processed routinely to paraffin wax. Human fetuses were obtained within 6 h following termination of pregnancy using Gemeprost vaginal pessaries. Fetal developmental age was carefully estimated based on size, including crown-heel, crown-rump and heel-toe measurements (Scammon and Calkins, 1929), menstrual history and ultrasound dating of pregnancy. Normality of fetuses was confirmed by autopsy. Blood samples (up to 1 ml) were obtained, by cardiac puncture, from fetuses 13–20 weeks gestation. The study was approved by the Paediatric/Reproductive Medicine Ethics of Medical Research Sub-Committee of Lothian Committee on Medical Res

Blood samples and cell normal female non-pregna deliveries. Five ml aliquots (Nycomed, Norway) in 15 Mononuclear cells at the p harvested using a Pasteur pi 5 ml of cold phosphate buf ethylenediaminetetra-acetic finally resuspended in PBS/

Slide preparation. Aliqu mixture of the two were cyt Formaldehyde-fixed paraffi

Immunocytochemistry. munocytochemical procedur gift from Prof. P. Berverley hemoglobin antibody at a Chemical & Scientific Corpo PAP (DAKO) at 1:100. All a and antibody incubations w

Dry cytospins were fixed saline (TBS). The standard I peroxidase activity was block were rinsed in two changes o in TBS for 5 min to block nc cytospins were incubated wit followed by incubation with rabbit anti-mouse IgG for 30 serum in TBS for 5 min follc two to three drops of 3,3-dia temperature for 7 min. The I TBS followed by the additior The slides were washed in ru solution (0.4 g copper sulphat tap water. In our initial studie were counterstained with Ma and checked using a light mi

Immunohistochemistry o technique (Sternberger et al., through graded alcohol and

Flourescence in situ hybr 3:1 (v/v) methanol: glacial a a fresh preparation of the sam for 90 sec. The slides were wa 85%, and 100% alcohol and s kit (Cytocell, Oxon., UK) co with fluoresceinisothiocyanat recommended by the manufa coverslip coated with the fluo presence of hybridisation fluk probe and target DNA which allowed to hybridise to the tar were mounted in antifade sol

Microscopy. Slides were a illuminator with mercury vapo for DAPI; 09 for FITC; and

nshirt-Ahlert et al., 1990; amada et al., 1993). These could provide a means of ;. Bianchi, 1994; Davies et method of detection were naternal blood by density ited cell sorting (Bianchi et t al., 1993) or some com- ure then analysed for the tion (PCR) (Wachtel et al., s et al., 1994).
g fetal nucleated red blood ly, discriminating between monoclonal antibody rais- recently been used for im- the only available specific ne will not unequivocally lls because of the possibili- hich is greatly exaggerated e therefore combined im- romosome probes to iden- nocytochemistry and FISH genotypic analysis of cells, anti-alkaline phosphatase r et al., 1992; Price et al., example, Price et al., 1992) lysis in patients with the chromosome 8 as a marker perties of the APAAP reac- enotype and genotype by

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wing termination of pregnancies ge, U. K.) and Gemeprost vaginal velopment and the approximate ological criteria (O'Rahilly and d in 10% buffered formaldehyde re obtained within 6 h following developmental age was carefully e measurements (Scammon and formality of fetuses was confirm- icture, from fetuses 13–20 weeks licine Ethics of Medical Research

Sub-Committee of Lothian Health Board, the Ethics Committee of Tayside Health Board and Tayside Committee on Medical Research Ethics.

Blood samples and cell preparation. Peripheral venous blood samples (EDTA) were obtained from normal female non-pregnant adults and umbilical cord blood samples from normal male full term deliveries. Five ml aliquots of blood were carefully layered over 3.5 ml aliquots of Polymorphoprep (Nycomed, Norway) in 15 ml tubes which were then spun at 500 g for 30 min at room temperature. Mononuclear cells at the plasma/Polymorphoprep interface (upper of the two bands obtained) were harvested using a Pasteur pipette and dispensed into a clean tube. The cells were washed three times using 5 ml of cold phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 5 mM ethylenediaminetetra-acetic acid (EDTA) followed by a 10 min spin at 400 g each time. The cell pellets were finally resuspended in PBS/BSA/EDTA at a concentration of 10^6 cells/ml.

Slide preparation. Aliquots (100 µl) of adult and cord blood mononuclear cells as well as a 1:1 (v/v) mixture of the two were cytocentrifuged onto glass slides (Cytospin, Shandon) and air dried overnight. Formaldehyde-fixed paraffin embedded embryonic tissue was cut at 3 µm sections.

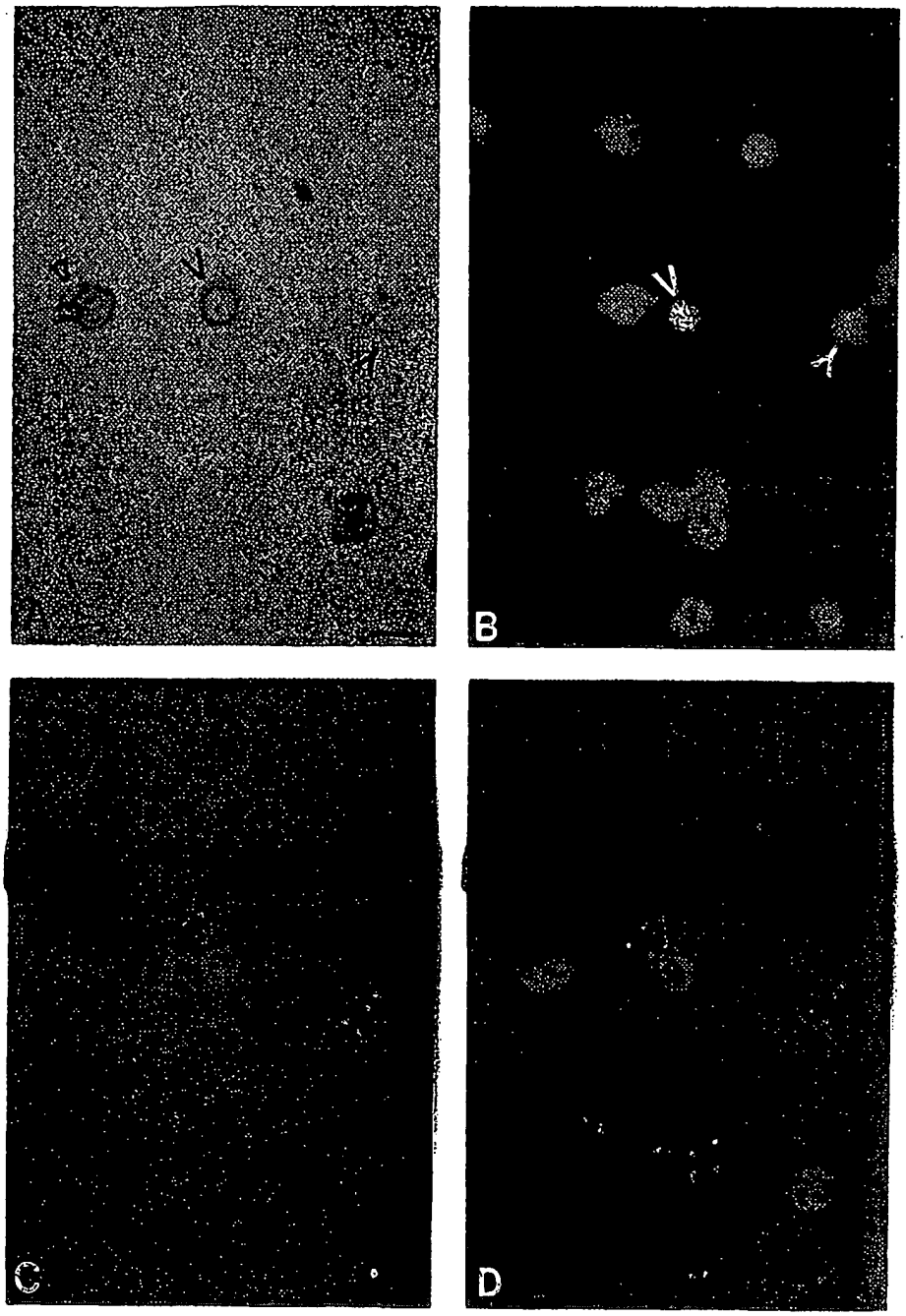
Immunocytochemistry. The following polyclonal and monoclonal antibodies were used in the immunocytochemical procedure: (a) UCHγ anti-human fetal hemoglobin antibody culture supernatant (a gift from Prof. P. Berverley, London, UK) at a 1:3 titre (2), (b) ε-1-276 anti-human embryonic hemoglobin antibody at a 1:200 titre for cytospins and 1:1000 for tissue sections (Accurate Chemical & Scientific Corporation, NY, USA) (c) rabbit anti-mouse IgG (DAKO) at 1:25, and (d) mouse PAP (DAKO) at 1:100. All antibodies were diluted in TBS containing 20% normal rabbit serum (Serotec) and antibody incubations were carried out in a humidified chamber at room temperature.

Dry cytospins were fixed in acetone for 3 min and washed for 5 min in two changes of Tris-buffered saline (TBS). The standard PAP technique (Sternberger et al., 1970) was modified. Briefly, endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide in TBS for 30 min after which time slides were rinsed in two changes of TBS for 5 min. Cytospins were incubated with 20% normal rabbit serum in TBS for 5 min to block non-specific binding sites. After removal of most of the normal rabbit serum, cytospins were incubated with UCHγ (or ε-1-276) for 30 min. After washing the slides in TBS as before, followed by incubation with 20% normal rabbit serum in TBS for 5 min, cytospins were incubated with rabbit anti-mouse IgG for 30 min. The slides were washed as above and incubated with 20% normal rabbit serum in TBS for 5 min followed by mouse PAP for 30 min. The slides were then washed as above and two to three drops of 3,3-diaminobenzidine (DAB) substrate solution were added and incubated at room temperature for 7 min. The DAB solution was prepared by dissolving 2.5 mg of DAB (Sigma) in 5 ml of TBS followed by the addition 0.1 ml of freshly prepared 1% hydrogen peroxide immediately before use. The slides were washed in running tap water for 2 min followed by a 5 min incubation in copper sulphate solution (0.4 g copper sulphate, 0.72 g sodium chloride, 100 ml distilled water) and rinsed again in running tap water. In our initial studies to check the morphology of the cells after immunocytochemistry, the slides were counterstained with Mayer's hematoxylin (Sigma) for 20 min, dehydrated through graded alcohols, and checked using a light microscope Zeiss Axioskop 20.

Immunohistochemistry on tissue sections was performed using UCHγ or ε-1-276 and a standard PAP technique (Sternberger et al., 1970). Sections were lightly counterstained with haemotoxylin, dehydrated through graded alcohol and cleared in xylene prior to coverslipping in synthetic resin.

Flourescence in situ hybridisation (FSH). Following immunocytochemistry, cytospins were fixed in 3:1 (v/v) methanol: glacial acetic acid for 30 min at room temperature. The fixation was repeated with a fresh preparation of the same fixative for a further 30 min and with 70:30 (v/v) glacial acetic acid: water for 90 sec. The slides were washed for 5 min in two changes of PBS and then dehydrated through 70%, 85%, and 100% alcohol and air-dried at room temperature. FISH was performed using a Chromoprobe-I kit (Cytocell, Oxon., UK) containing centromere-specific probes for chromosomes X (directly labelled with fluoresceinisothiocyanate (FITC)) and Y (directly labelled with cyanine (Cy3)) using the procedure recommended by the manufacturer. Briefly, hybridisation fluid was pipetted onto each cytospin and a coverslip coated with the fluorescent probes was placed on top and sealed with rubber solution. In the presence of hybridisation fluid, the probes were eluted from the coverslip and after denaturation of both probe and target DNA which occurred under the coverslip by heating the slides to 70 °C, the probes were allowed to hybridise to the targets for 25 min at 37 °C. After several post hybridisation washes the slides were mounted in antifade solution containing diamidino-2-phenyl-indole dihydrochloride (DAPI).

Microscopy. Slides were analysed using a Zeiss Axioskop 20 microscope equipped with a microscope illuminator with mercury vapour short-arc lamp HBO 50W and appropriate Zeiss filter combinations (02 for DAPI; 09 for FITC; and 15 for TRITC/CY3). The immunopositive cells were located using the



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microscope in the visible light mode, after which the visible light source was blocked and the microscope switched to fluorescense mode and slides analysed using each filter set. Cells were photographed on Fujichrome Provia 400 colour film (Fuji Photo Film Co., Tokyo, Japan) both in visible light mode and fluorescence mode. Black and white negatives and prints were then made from the colour film.

Results

In sections of fixed embryonic tissue intense immunoreactivity was detected, using a monoclonal antibody raised against human embryonic hemoglobin, in all nucleated and non-nucleated red blood cells. In fetal blood samples 13–20 weeks gestation only a few nucleated red blood cells show significant expression of embryonic haemoglobin (range 1:7 to 1:1000 positive to negative nucleated cells in individual fetal blood samples). There were a higher number of immunopositive erythrocytes, compared to nucleated red cells. Within the erythrocyte population, the abundance of intensely immunopositive cells at 14 weeks gestation was found to be approximately 1:20 whereas by 20 weeks this ratio had decreased to approximately 1:90 and by term only a very occasional cell was positive. Although this monoclonal human embryonic hemoglobin antibody has potential as a specific early gestation erythropoetic marker, our experiments to establish a new technique were conducted using term fetal blood for a variety of ethical and practical reasons. The UCHγ antibody was therefore used as it is a better marker later in gestation.

In preliminary experiments to establish the conditions for anti-UCHγ immunocytochemistry, eosinophils were found to produce strong non-specific immunostaining in both test and negative controls as they contain significant endogenous peroxidase which is not completely blocked by the routine pre-treatment with hydrogen peroxide prior to the PAP procedure. This was not a problem in our combined immuno-FISH procedure after the Polymorphoprep separation as the visualisation of eosinophils post-FISH was extremely rare.

In the immunocytochemical procedure, using mixed adult female and male umbilical cord blood, background staining was minimal (Fig. 1 A). Anti-UCHγ immunopositive and negative cells were easy to differentiate even in slides post-FISH (Fig. 1 A). In addition post-FISH leucocyte nuclei are seen as background shadows (Fig. 1 A). DAPI staining (Fig. 1 B) shows the location of nucleated cells, the intensity of staining increasing with increasing condensation of chromatin. This is particularly obvious in the FNRBC in the centre of Fig. 1 B.

Y-chromosome FISH signals were found to be clear and bright after our combined immuno-FISH procedure with no interfering background in either fetal leucocytes or FNRBCs (Fig. 1 C). X-chromosome FISH signals (Fig. 1 D) were also bright but there was background fluorescence in the FNRBCs and to a lesser extent in non-nucleated red blood cells (Fig. 1 D).

When APAAP immunocytochemistry was initially used instead of the PAP technique and FISH carried out subsequently, nuclei were found to be damaged as shown in Fig. 2. Very few X- or Y- chromosome signals were observed on these slides (results not shown) and the immunostain was substantially reduced by the FISH procedure.

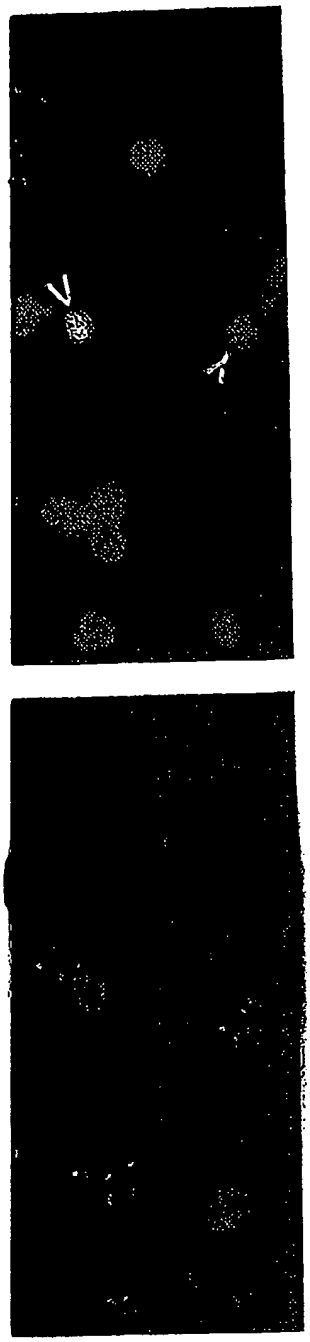

Fig. 1. A single field of a mononuclear cell preparation of mixed adult female and male umbilical blood showing: A) Immunocytochemistry of UCHγ showing reactive nucleated (V) and non-nucleated (Δ) red blood cells with leucocyte nuclei as background shadows (e. g. Y); B) DAPI staining showing nuclei with intense reactivity where chromatin is condensed (V, same cell as in A) and also in leucocytes (Y, same cell as in A); C) FISH showing Y-chromosomal signals in nucleated red blood cell (V, same cell as in A & B) and in fetal leucocytes (e. g. Y, same cell as in A and B); D) FISH showing intense point X-chromosomal signals (e. g. in leucocyte Y, same cell as in A, B and C) and diffuse background fluorescence in red blood cells (e. g. FNRBC V, same cell as in A, B and C). Bar = 5 μm.

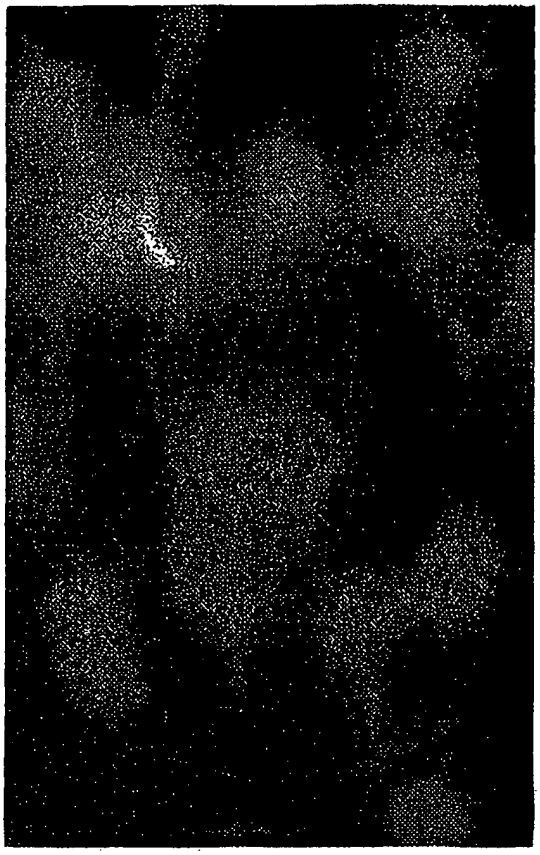

Fig. 2. A DAPI stain of a mononuclear cell preparation of mixed adult female and male umbilical blood showing multiple damaged nuclei.

Discussion

A technique of combined immunocytochemistry and FISH on solid tissues has been used by other investigators for several purposes, for example simultaneous detection of cell cycle, genomic and phenotypic parameters of tumour cells (Speel et al., 1994), study of lineage involvement in patients with myelodysplastic syndromes (Kibbelaar et al., 1992) identification of cell type infected by human parvovirus (Porter et al., 1990), and characterisation of tumour cells (Webert-Matthiesen et al., 1992) and on adult blood cells to discriminate between donor and recipient peripheral blood cells in bone marrow and cardiac transplantation (van den Berg et al., 1991; Hruban et al., 1993). However, using conventional immunocytochemistry and FISH, dual detection of cytoplasmic or endoplasmic reticulum membrane proteins and/or cytoplasmic RNA in combination with chromosomal hybridisation is not feasible with normal interphase in situ methods because a prerequisite is the removal of both the cytoplasm and membranes of the cell to allow more efficient nuclear hybridisation (Price et al., 1992). Price et al. (1992) have therefore developed a modified APAAP-FISH procedure to avoid the methanol/acetic

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acid fixation normally required for hybridisation for use in adult peripheral blood cells from patients with polycythemia vera. Unfortunately, their method uses Fast Red which forms an end product that is soluble in organic solvents (Fast Red Substrate System data sheet, Dako), whereas all FISH methods require the use of organic solvents, e.g. 70% formamide at 70 °C. More recently, Zheng et al., 1993 have applied this modified combined APAAP-FISH technique using confocal laser scanning microscopy to FNRBCs isolated from maternal blood and using this technique they found that laboratory methods could be completed within three days after peripheral blood sampling. We therefore initially tried to use a combined APAAP-FISH technique but we found that almost all the Fast Red reaction product was lost during the FISH, leaving variable and patchy staining. In addition, nuclear damage was consistently-observed, e.g. Fig. 2. Moreover, penetration of our X- and Y-centromere probes was poor, even after repeated methanol: acetic acid (3:1) as well as an additional acetic acid: water (70:30) fixation which we found in separate FISH experiments to be necessary for probe penetration and for consistent and uniform hybridisation.

For simple routine non-quantitative immunostaining of FNRBCs, in combination with diagnostic FISH, it is most useful to visualise the developed immunoreactive staining and the FISH signals alternately on the same cell without changing microscope or slide position (see Methods). This obviates the need for the time consuming spatial orientations and light microscope photography between the two visualisation procedures and subsequent relocation using grid positioning followed by analysis using confocal laser scanning microscopy (Zheng et al., 1993). Moreover to enable us to develop more complex methods for the subsequent quantitation of protein and/or mRNA expression in FNRBCs, for example using image analysis, a stable chromogenic product is essential. DAB is a sensitive and stable chromogen and resistant to organic elution in immunopositive cells and the logical alternative to Fast Red. We therefore developed a PAP method using DAB for immunological detection prior to FISH and reduced the complete procedure time to 6–7 h. The DAB reaction product in the immunopositive fetal nucleated and non-nucleated red blood cells was preserved throughout the FISH procedure which enabled us to alternately visualise immunostaining and FISH signals. The Y-chromosome probe gave a clear intense signal in the fetal cells with no background staining allowing us to unequivocally identify immunopositive cells as fetal in origin. The X-chromosome probe produced two nuclear signals in the maternal nucleated cells and one in the male fetal nucleated cells, and background cytoplasmic and nuclear fluorescence, although present, did not interfere with the visualisation of the intense nuclear signal of the X probe (see Fig. 1D).

We have previously shown that a predominantly hepatic protein in adults (glucose-6-phosphatase) is expressed in nucleated red blood cells in the developing embryo and early fetus (Hume et al., 1995). We are now using the combined immunocytochemistry-FISH to explore the potential for diagnosis of disorders of fetal hepatic gene expression early in pregnancy.

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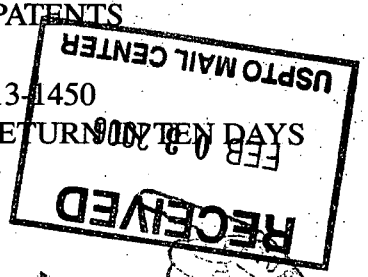

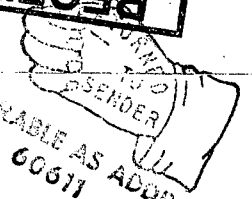

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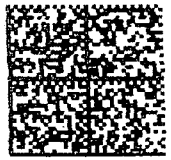

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